

ApoA-II modulates the association of HDL with class B scavenger receptors SR-BI and CD36

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Abstract The class B scavenger receptors SR-BI and CD36 exhibit a broad ligand binding specificity. SR-BI is well characterized as a HDL receptor that mediates selective cholesteryl ester uptake from HDL. CD36, a receptor for oxidized LDL, also binds HDL and mediates selective cholesteryl ester uptake, although much less efficiently than SR-BI. Apolipoprotein A-II (apoA-II), the second most abundant HDL protein, is considered to be proatherogenic, but the underlying mechanisms are unclear. We previously showed that apoA-II modulates SR-BI-dependent binding and selective uptake of cholesteryl ester from reconstituted HDL. To investigate the effect of apoA-II in naturally occurring HDL on these processes, we compared HDL without apoA-II (from apoA-II null mice) with HDLs containing differing amounts of apoA-II (from C57BL/6 mice and transgenic mice expressing a mouse apoA-II transgene). The level of apoA-II in HDL was inversely correlated with HDL binding and selective cholesteryl ester uptake by both scavenger receptors, particularly CD36. Interestingly, for HDL lacking apoA-II, the efficiency with which CD36 mediated selective uptake reached a level similar to that of SR-BI. These results demonstrate that apoA-II exerts a marked effect on HDL binding and selective lipid uptake by the class B scavenger receptors and establishes a potentially important relationship between apoA-II and CD36.—de Beer, M. C., L. W. Castellani, L. Cai, A. J. Stromberg, F. C. de Beer, and D. R. van der Westhuyzen. ApoA-II modulates the association of HDL with class B scavenger receptors SR-BI and CD36. *J. Lipid Res.* 2004. 45: 706–715.

Supplementary key words lipoprotein metabolism • selective lipid uptake • atherosclerosis • apolipoprotein A-II • high density lipoprotein

HDL levels are inversely related to the risk of atherosclerotic disease (1, 2). The atheroprotective effects of HDL appear to include its role in reverse cholesterol transport, a pathway by which unesterified cholesterol

from peripheral cells is sequestered by plasma HDL, esterified by lecithin:cholesterol acyltransferase, and then delivered to hepatocytes by a process of selective lipid uptake in which cholesteryl esters (CEs) are taken up without concomitant apolipoprotein uptake (3, 4). HDL consists of a heterogeneous mixture of particles that vary in size, density, and composition (5) and contains particles with both apolipoprotein A-I (apoA-I) and apoA-II (LpA-I/A-II) and those with apoA-I but not apoA-II (LpA-I). ApoA-II is the second most abundant protein in HDL; however, its physiological role remains unclear (6, 7). ApoA-II plays a key role in regulating HDL concentration and particle size and is often positively associated with plasma FFA and triglyceride levels (5). Interestingly, the majority of studies have indicated that apoA-II functions in a “proatherogenic” manner. Transgenic mice overexpressing mouse apoA-II showed increased susceptibility to atherosclerosis (8). These mice have increased levels of large CE-rich HDL, increased plasma triglyceride-rich lipoproteins, and insulin resistance. The proatherogenic effect of apoA-II may be caused by its negative effects on reverse cholesterol transport or by the high levels of triglyceride-rich lipoproteins. ApoA-II is known to negatively affect a number of processes involved in reverse cholesterol transport, including the activities of hepatic lipase, lipoprotein lipase, CE transfer protein (CETP), and lecithin:cholesterol acyltransferase, as well as cellular cholesterol efflux and scavenger receptor class B type I (SR-BI)-mediated HDL binding and selective uptake (6, 7). In contrast to transgenic mice, apoA-II-deficient mice have low HDL levels, increased clearance of triglyceride-rich lipoproteins, and insulin hypersensitivity (9). The majority of

Abbreviations: apoA-II, apolipoprotein A-II; CE, cholesteryl ester; oxLDL, oxidized LDL; POPC, 1- α -palmitoyllecithin; rHDL, reconstituted HDL; SR-BI, scavenger receptor class B, type I.

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Manuscript received 2 October 2003 and in revised form 24 December 2003.

Published, JLR Papers in Press, January 16, 2004.

DOI 10.1194/jlr.M300417JLR200

studies using human or mouse apoA-II transgenic mice also indicated apoA-II to be proatherogenic (8, 10–13), although one indicated an antiatherogenic effect (14). Structural differences between human apoA-II (a dimer) and mouse apoA-II (a monomer) may explain many of the observed differences between the biological properties of the two apoA-II species (7). For example, human and mouse apoA-II in transgenic mice have opposite effects on HDL size, apoA-I content, plasma concentration, and protection from oxidation (6, 7, 14).

The scavenger receptors SR-BI and CD36 are closely related members of the class B scavenger receptor family of proteins. SR-BI plays a key role in HDL metabolism, binding HDL and facilitating selective CE uptake into cells, most prominently hepatocytes and steroidogenic cells (15, 16). SR-BI has a broad ligand specificity and in addition to binding HDL also binds native as well as oxidized LDL (oxLDL), VLDL, anionic phospholipids, and either free or lipid-bound apolipoproteins such as apoA-I, apoA-II, and apoC-III (15). Evidence indicates that human apoA-II decreases HDL binding and selective lipid uptake. LpA-I/A-II particles exhibit lower SR-BI-specific binding and selective lipid uptake than LpA-I (17), and apoA-II enrichment of HDL in vitro also decreases selective uptake in an adrenal cell line (18). Using reconstituted particles, we previously showed that apoA-II decreased SR-BI-specific particle binding (19). These findings are in agreement with studies showing more rapid catabolism of apoA-I in human LpA-I than in LpA-I/A-II (20) and more rapid biliary secretion of CE in rats from LpA-I than from LpA-I/A-II (21).

The scavenger receptor CD36 is expressed in a wide range of cell types, including platelets, adipocytes, cardiac and skeletal muscle cells, microvascular endothelial cells, and macrophages (22). Like SR-BI, it binds a variety of ligands, including anionic phospholipids, oxLDL, and HDL, as well as long chain fatty acids, collagen, and thrombospondin (22). Despite high-affinity binding of HDL, CD36 mediates selective CE uptake with low efficiency compared with SR-BI (23, 24). CD36 has been implicated in a broad range of biological functions, such as the uptake of oxLDL, the clearance of apoptotic cells, the regulation of the antiangiogenic effect of thrombospondin-1, and the uptake of fatty acids (22). CD36 null mice have increased plasma levels of cholesterol, triacylglycerol, and fatty acids and altered glucose metabolism (25), supporting other lines of evidence that CD36 has a key role in fatty acid transport and lipid metabolism and indirectly in glucose metabolism and insulin resistance (22). Interestingly, two genetically diverse animal models, the spontaneously hypertensive rat, which lacks functional CD36 (26), and the transgenic mouse overexpressing mouse apoA-II (27), share the common phenotypes of abnormal plasma lipoproteins and insulin resistance. CD36 null mice do not develop insulin resistance but, as mentioned above, do have altered lipid and glucose metabolism (25). These findings suggest the possibility of a direct link between CD36 and apoA-II in this insulin-resistant phenotype.

In the present study, we have investigated the effect of mouse apoA-II on the interaction of HDL with the two class B scavenger receptors using HDL isolated from control C57BL/6 mice, transgenic mice overexpressing mouse apoA-II, or apoA-II null mice. Studies addressing the effects of mouse apoA-II on HDL interaction with SR-BI have not been described previously. The effect of apoA-II on HDL interaction with CD36 is also addressed for the first time. HDLs containing different amounts of apoA-II were shown to differ markedly in their ability to bind CD36 and also to deliver CE selectively to cells in a CD36-dependent manner.

EXPERIMENTAL PROCEDURES

Mice

Transgenic mice containing multiple copies of the mouse apoA-II gene were derived as described (8). C57BL/6 mice were purchased from Jackson Laboratories, and mice deficient in apoA-II were kindly provided by Dr. J. L. Breslow (28). Animals were maintained in compliance with Department of Veterans Affairs Institutional Animal Care and Use Committee guidelines.

HDL isolation

HDL ($d = 1.063$ – 1.21 g/ml) was isolated from fresh pooled mouse plasma (10–20 mice per pool) by sequential ultracentrifugation, dialyzed against 150 mM NaCl and 0.01% EDTA, pH 7.4, sterile-filtered through 0.2 μ m filters (Millipore), and stored under nitrogen at 4°C as previously described (29). Protein was quantified by the method of Lowry et al. (30), and lipid compositions were determined enzymatically (WAKO Chemicals, Osaka, Japan). The composition of each pool of HDL of the same type was similar to the composition shown in Table 1. The sizes of the HDLs were compared by nondenaturing gel electrophoresis using a 4–18% gradient gel.

LDL isolation and oxidation

LDL ($d = 1.019$ – 1.063 g/ml) was isolated from fresh normal human plasma by sequential ultracentrifugation (29) and oxidized with 5 μ M CuSO₄ (24). OxLDL had an average electrophoretic mobility of 1.4 and exhibited extensive aggregation of apoB as visualized by SDS-PAGE.

Radiolabeling of lipoproteins

Lipoproteins were double labeled by iodination of the protein component (31) and tracing the CE with nonhydrolyzable, intra-

TABLE 1. HDL composition

Sample	C57HDL (n = 3)	AIItrHDL (n = 3)	AII-/-HDL (n = 3)
Protein	52.0 \pm 3.6	52.0 \pm 1.0	54.3 \pm 3.1
Total cholesterol	18.0 \pm 1.7	19.0 \pm 1.7	12.7 \pm 2.1
Free cholesterol	4.0 \pm 1.0	2.7 \pm 0.6	4.7 \pm 0.6
Cholesteryl ester	14.0 \pm 2.0	16.3 \pm 2.1	8.0 \pm 2.6
Phospholipid	28.7 \pm 3.1	28.0 \pm 1.0	29.7 \pm 5.5
Triglyceride	1.3 \pm 0.6	1.0 \pm 0.0	3.3 \pm 1.5

Values shown are percentages of mass and represent means \pm SEM of three separate preparations for each HDL type. AIItrHDL, HDL from apolipoprotein A-II (apoA-II) transgenic mice; AII-/-HDL, HDL from apoA-II null mice; C57HDL, control HDL from C57BL/6 mice.

cellularly trapped [$1\alpha,2\alpha(n)^3\text{H}$]cholesteryl oleyl ether (32). Briefly, [$1\alpha,2\alpha(n)^3\text{H}$]cholesteryl oleyl ether suspended in dichloromethane was coated onto the inside of a glass tube by evaporation under nitrogen. Partially purified CETP was used to transfer the CE to iodinated lipoproteins. Double-labeled lipoproteins were separated from CETP by ultracentrifugal flotation at densities of 1.063 g/ml (OxLDL) and 1.21 g/ml (HDL). The integrity of all of the labeled lipoprotein ligands was verified by SDS-PAGE and gradient gel electrophoresis.

Preparation of reconstituted HDL

The protein concentrations of lipid-free apoA-I and apoA-II were determined as the average of concentrations measured by the Lowry assay (33) and absorbance at 280 nm, using an extinction coefficient of 1.13 ml/mg/cm for apoA-I, or at 276 nm, using an extinction coefficient of 0.69 ml/mg/cm for apoA-II (34). Reconstituted HDLs (rHDLs) containing α -phosphatidylcholine (POPC) were prepared by the sodium cholate dialysis method as described (19, 35). Briefly, rHDLs containing human apoA-I were prepared with molar ratios of 1:5:95 (apoA-I/free cholesterol/POPC). The purity and size of rHDL were examined on 8–25% gradient gels under nondenaturing conditions using the Amersham Pharmacia Biotech Phast System. The diameters of the rHDL particles were ~ 100 Å, and particles were more than 95% homogeneous in size. Chemical cross-linking with bis(sulfosuccinimidyl)suberate determined that AI-rHDL contained two molecules of apoA-I. Experiments with AI-rHDL were performed within 20 days of particle preparation to avoid time-dependent size rearrangement of particles. Hybrid AI/AII-rHDL was prepared by incubating lipid-free human apoA-II with the AI-rHDL at a molar ratio of 2:1 (AI:AII) for at least 20 min at an ambient temperature. This resulted in one dimeric apoA-II molecule on each rHDL. These particles were used within 24 h.

Cell culture

COS-7 cells were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. CHO cells (clone ldlA7) that lack the LDL receptor (provided by M. Krieger) as well as CHO cells stably transfected with SR-BI or CD36 were cultured in Ham's F12 medium containing 5% (v/v) FBS, 2 mM glutamine, 50 U/ml penicillin, and 50 $\mu\text{g}/\text{ml}$ streptomycin (36).

Preparation of adenoviral vectors

Sequences encoding mouse CD36 or SR-BI were inserted into the adenovirus expression vector pAdCMV.link (37) containing the cytomegalovirus immediate-early enhancer promoter element to yield pAdCD36 or pAdSR-BI. The expression plasmids were cotransfected into 293 cells with replication-defective adenoviral DNA as described previously (37) to generate recombinant adenoviruses expressing mCD36 or mSR-BI. Adnull (provided by Dr. D. J. Rader) is a recombinant virus with analogous adenoviral sequences containing no transgene.

Western blot analysis

The expression of scavenger receptors administered to COS cells via adenoviral vector was verified in cell lysates by Western blot analysis as described using anti-mCD36 (24) and anti-mSR-BI (36) antibodies.

Ligand binding and uptake assays

COS and CHO cells were seeded on 12-well plates 48 h before assay (1×10^5 cells per well). Preliminary experiments with adenovirus expressing Green Fluorescent Protein were performed to determine that adenoviral gene transfer occurred in more than 95% of treated cells. Adenoviral vector-mediated gene over-

expression in COS cells was performed by the addition of Adnull, AdCD36, or AdSR-BI at a multiplicity of infection of 2,000 particles per cell, except where otherwise indicated, 24 h before assay. Cell association assays were described previously (36) and for COS cells were performed in triplicate at 37°C in DMEM supplemented with 0.5% essentially fatty acid-free BSA, 100 U/ml penicillin and streptomycin, and radiolabeled lipoprotein. To compare the relative expression levels of CD36 and SR-BI at the cell surface, COS-CD36 and COS-SR-BI cells were incubated with oxLDL (10 $\mu\text{g}/\text{ml}$) for 2 h at 4°C to determine ligand binding at the cell surface. CHO cell association assays were performed in triplicate at 37°C in Ham's F12 medium containing 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM glutamine, 0.5% essentially fatty acid-free BSA, and radiolabeled lipoprotein (19). After incubating for the indicated times, unbound ligand was removed from cells by washing four times with 50 mM Tris-HCl and 150 mM NaCl, pH 7.4, containing 2 mg/ml fatty acid-free BSA followed by two washes in the same buffer without BSA. All washes were performed at 4°C with prechilled solutions. Cells were solubilized in 0.1 N NaOH for 60 min at room temperature before protein and radioactivity quantitation. Receptor-specific cell association values were calculated as the difference between the total for AdCD36 and AdSR-BI-expressing cells and corresponding values for Adnull control cells. Selective uptake expressed in terms of nanograms of CE was calculated by subtracting the amount of bound CE (calculated from the ^{125}I cell-associated radioactivity) from the total amount of cell-associated CE. Noniodide TCA-soluble degradation products were measured in the culture medium and in all cases were less than 20% of the cell-associated material. K_d values were determined by nonlinear regression analysis of receptor-specific cell association values using Prism® software (GraphPad Software, San Diego, CA).

Statistical analysis

Cell association and selective uptake of HDL from apoA-II null, C57BL/6, and apoA-II transgenic mice were compared by two-way ANOVA to control for an experimental effect in experiments carried out on different days using different sets of ligands. The efficiencies with which the three HDLs served as ligands for selective uptake, calculated as the amount of selective uptake relative to HDL associated with the cells, were compared by paired *t*-test to control for an experimental day effect. Statistical significance was set at $P < 0.05$. Values are expressed as means \pm SEM.

RESULTS

To investigate the influence of mouse apoA-II on HDL binding and uptake by SR-BI and CD36, we compared mouse HDL without apoA-II (from apoA-II null mice) with HDL containing varying amounts of apoA-II (from C57BL/6 and murine apoA-II transgenic mice). In these studies, we used a homologous system in which mouse HDLs were compared in their interaction with mouse SR-BI and CD36. The apolipoprotein content of these ligands is shown in Fig. 1A. ApoA-II was absent in HDL from apoA-II null mice (AII $^{-/-}$), whereas the ratio of apoA-II to apoA-I was ~ 3 -fold greater in HDL from apoA-II transgenic mice (AII tr) than in control HDL from C57BL/6 mice (C57), as reported previously (8). Other apolipoproteins, including apoA-IV and apoE, did not vary significantly in amount between the different HDLs. Minor

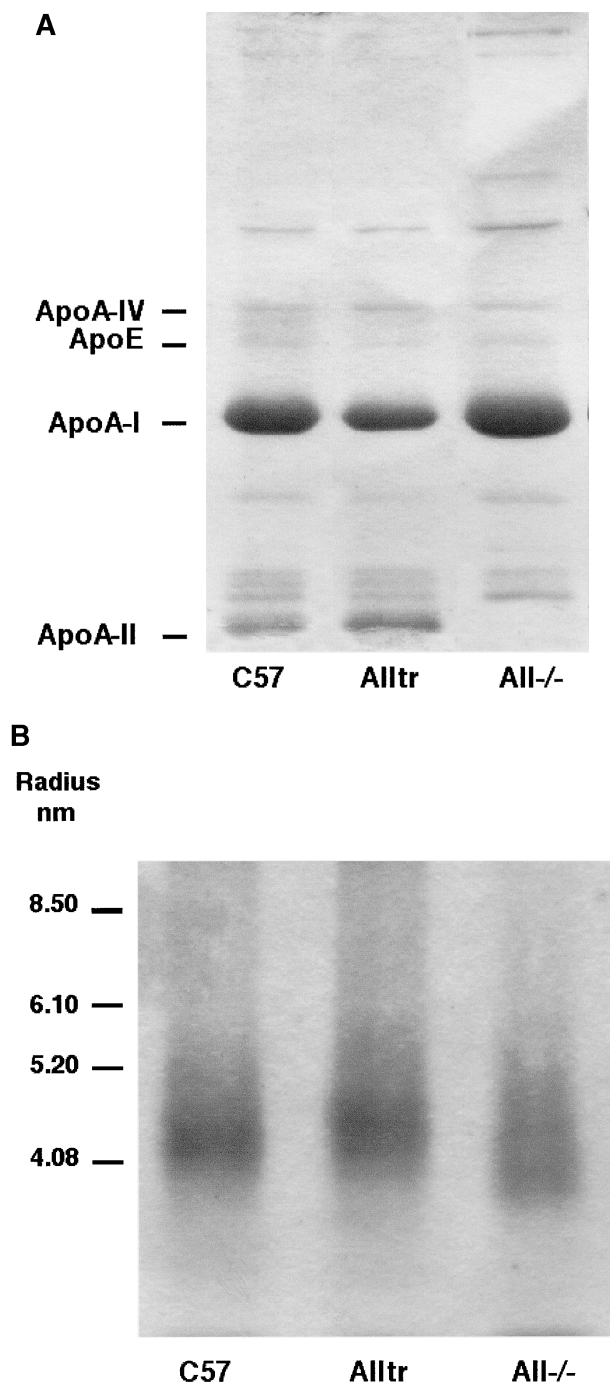


Fig. 1. A: SDS-PAGE analysis of mouse HDLs. The Coomassie blue stain of HDL apolipoproteins separated by SDS-PAGE on a 5–20% acrylamide gradient gel shows the lack of apolipoprotein A-II (apoA-II) in apoA-II null mice (AII-/-) and the increased ratio of apoA-II to apoA-I in apoA-II transgenic mice (Alltr) compared with C57BL/6 mice (C57). HDL loading was 5 μ g per lane. B: Nondenaturing gel electrophoresis of mouse HDLs. Mouse HDLs analyzed by electrophoresis on a 4–18% nondenaturing gel were stained with Coomassie blue. HDL loading was 5 μ g per lane. Stokes radii of molecular size markers are indicated at left.

bands corresponding to apoB proteins were seen in this but not other sets of ligand preparations. The HDLs also showed differences in size as determined by nondenaturing gradient gel electrophoresis (Fig. 1B). AlltrHDL was

slightly larger than C57HDL, which in turn was larger than AII-/-HDL, in agreement with previous reports of AlltrHDL (38) and AII-/-HDL (9). The composition of the particles is shown in Table 1. The protein content (percentage of mass) of the three HDLs was similar, as was the phospholipid content. There was a significantly reduced level of CE in AII-/-HDL, \sim 2-fold lower than in the other two ligands, and reduced free cholesterol content in AlltrHDL. The triglyceride content was \sim 3-fold higher in the AII-/-HDL than in the other two ligands.

The three HDLs were compared as ligands for binding to SR-BI and SR-BI-dependent selective lipid uptake. Concentration-dependent association and selective CE uptake were determined at 37°C for 2 h with 125 I/ 3 H double-labeled HDLs in CHO cells stably transfected with mouse SR-BI (CHO-SRBI). SR-BI-specific values were calculated as the difference between the values in CHO-SRBI and untransfected control CHO cells. Degradation of HDL ligands after 2 h of incubation was relatively low (less than 15% of cell-associated ligand), indicating a low rate of receptor-dependent ligand endocytosis and degradation. High-affinity, saturable, and SR-BI-specific association was observed for all three ligands (Fig. 2A). Given the similar protein composition (percentage of total mass) of the three HDLs and their similar sizes, the amount of cell-associated protein reflects the approximate number of associated HDL particles. AII-/-HDL exhibited the greatest SR-BI association (apparent B_{max} = 955 \pm 24 ng/mg cell protein), and this value decreased with increasing apoA-II content of the HDL (apparent B_{max} = 878 \pm 12 and 729 \pm 16 ng/mg cell protein for C57HDL and AlltrHDL, respectively). In three separate experiments, no significant differences were observed between the apparent affinities of SR-BI-specific association of the different HDLs (apparent K_d = 10.4 \pm 1.5, 15.3 \pm 4.8, and 10.7 \pm 0.8 μ g/ml HDL protein for AII-/-HDL, C57HDL, and AlltrHDL, respectively). The affinities of ligand/receptor interaction were similar to those we reported previously for mouse HDL and higher than the affinity reported for human HDL binding to mouse or human SR-BI (K_d from 15 to 44 μ g/ml) (15, 39–41).

SR-BI-mediated uptake of CE from the three HDLs is shown in Fig. 2B. As expected, significant SR-BI-mediated selective uptake was observed from each of the ligands. Selective uptake was calculated as the amount of cell-associated [3 H]CE that could not be accounted for by whole particle uptake (assessed by 125 I-HDL association). Selective CE uptake was greatest in the case of AII-/-HDL and lowest for AlltrHDL. Selective uptake of [3 H]CE at a ligand concentration of 70 μ g/ml was \sim 70% higher from AII-/-HDL than from AlltrHDL. These results indicate that SR-BI-specific HDL association and selective lipid uptake were inversely related to apoA-II content. Differences in selective uptake correlated closely with the differences in ligand association; therefore, when the efficiency of selective uptake was calculated as the amount of selective uptake expressed as a function of cell-associated HDL, no significant differences were observed between the different HDLs (data not shown). This indicates that the rate of

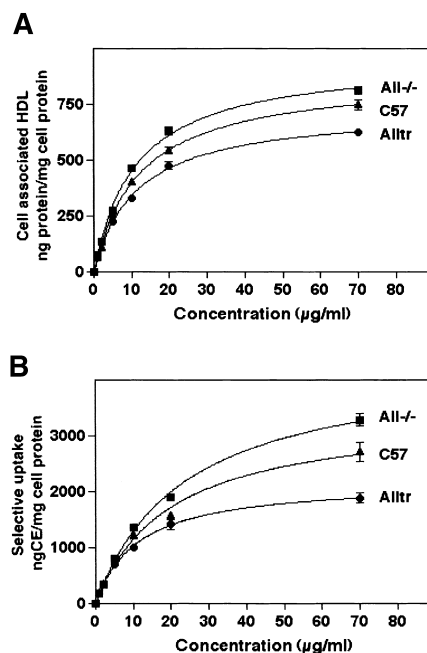


Fig. 2. Concentration-dependent association of mouse HDLs with mouse scavenger receptor class B type I (SR-BI). CHO cells stably transfected with mouse SR-BI were incubated for 2 h at 37°C with the indicated concentrations of ^{125}I / ^3H -labeled HDLs, and the cell-associated label was quantified as described in Experimental Procedures. Shown are SR-BI-specific cell association values calculated as the difference between values for SR-BI-transfected cells and nontransfected CHO ldlA7 cells. A: Cell-associated ^{125}I AII-/-HDL, C57HDL, and AIItrHDL. B: Selective uptake of cholesteryl ester (CE) from AII-/-HDL, C57HDL, and AIItrHDL. Selective uptake expressed as nanograms of CE was calculated by subtracting the amount of bound CE (calculated from the ^{125}I cell-associated radioactivity) from the total amount of cell-associated CE. Values represent means \pm SEM of triplicate determinations. Similar results were obtained in two additional experiments performed with two different sets of ligands.

CE uptake per bound particle is similar for the three HDLs. The similar efficiency of selective uptake for AII-/-HDL compared with C57HDL indicates that the lower CE content of AII-/-HDL does not become limiting to the CE uptake process. Although AII-/-HDL has the lowest CE content of the three particles, it allowed for the greatest CE uptake. The rate of selective uptake can also be considered in relation to the amount of HDL CE bound to SR-BI. This provides a measure of the fractional uptake rate for HDL CE by SR-BI. Because AII-/-HDL has an ~ 2 -fold lower CE content than AIItrHDL and C57HDL, the fractional uptake rate is ~ 2 -fold greater for AII-/-HDL than for the other two ligands.

The class B scavenger receptor CD36, like SR-BI, binds HDL with high affinity. Two cell systems were investigated to study HDL binding and selective lipid uptake by mouse CD36: stably transfected CHO cells expressing CD36 and COS cells expressing CD36 by adenovirus-mediated gene transfer. In both systems, a high-level expression of CD36 was achieved (Fig. 3A). Surprisingly, despite high CD36 expression in CHO-CD36 cells, very low levels of CD36-specific cell

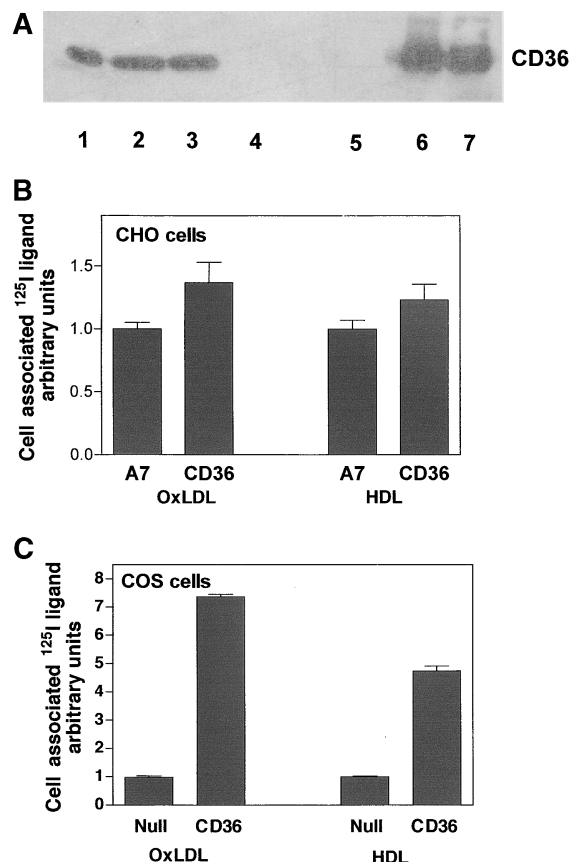


Fig. 3. CD36-dependent cell association of oxidized LDL (oxLDL) and HDL. A: CHO cells stably transfected with mouse CD36, as well as COS cells overexpressing mouse CD36 through adenoviral vector-mediated gene transfer, were subjected to immunoblot analysis. COS cells were treated with AdCD36 or control Adnull virus at a multiplicity of infection of 1,000 virus particles per cell. Cell lysates were obtained from duplicate wells. Lane 1, mouse adipose tissue. Lanes 2 and 3, CHO-CD36 cells. Lane 4, CHO-ldlA7 cells. Lane 5, COS cells treated with Adnull. Lanes 6 and 7, COS cells treated with AdCD36. (Adipose tissue, 5 μg protein/lane; cell lysates, 10 μg protein/lane.) B: CHO-CD36 cells were incubated with ^{125}I -labeled human oxLDL (10 $\mu\text{g}/\text{ml}$) or HDL₃ (20 $\mu\text{g}/\text{ml}$) for 1 h at 37°C. The association to CHO-CD36 cells is shown relative to that of control CHO-ldlA7 cells that were assigned an arbitrary value of 1. Similar HDL binding data were obtained in a separate experiment with a different batch of HDL₃. C: COS cells were treated with AdCD36 or control Adnull and incubated with ^{125}I -labeled human oxLDL (10 $\mu\text{g}/\text{ml}$) or HDL₃ (10 $\mu\text{g}/\text{ml}$) for 1 h at 37°C. The binding to CD36-expressing cells is shown relative to that of control Adnull cells that were assigned an arbitrary value of 1. Values represent means \pm SEM of triplicate (HDL) or quadruplicate (oxLDL) determinations. Data are representative of five experiments performed with five different batches of ligand.

association of either oxLDL, a known CD36 ligand, or mouse HDL was observed (Fig. 3B). In contrast, significant levels of CD36-specific association of both oxLDL and HDL were obtained in COS cells overexpressing this receptor (Fig. 3C). CD36-specific values were calculated as the difference in values obtained in cells treated with either AdCD36 or Adnull, an adenoviral vector that does not encode a functional protein. The reason for the lack of functional expression of CD36 in the transfected CHO cells is unclear.

The CD36-dependent association and selective lipid uptake of the different murine HDLs were then analyzed in the overexpressing COS cells. Unexpectedly large differences in cell association were observed between the three ligands (**Fig. 4A**). As in the case of SR-BI, high-affinity receptor association at 37°C was observed for each ligand, with the AII-/-HDL exhibiting the highest cell association and AIItrHDL the lowest. In five experiments performed with five different sets of labeled HDL, the maximal association of AII-/-HDL exceeded that of AIItrHDL by ~3.0-fold (**Table 2**). Although the difference between the apparent B_{max} values for AII-/-HDL and C57HDL was not significant, the binding values calculated at a ligand concentration of 60 $\mu\text{g/ml}$ were statistically different between each of the ligands (data not shown). The apparent affinities of the interaction of the three ligands with CD36 did not differ significantly from each other (**Table 2**). Therefore, the differences in the association of the ligands were the result of significant differences in the apparent B_{max} values of the ligands. The differences be-

TABLE 2. HDL association with CD36

HDL	Apparent K_d (n = 5) $\mu\text{g/ml}$	B_{max} (n = 5) ng/mg cell protein
AII-/-HDL	28.4 \pm 2.4	587 \pm 129
C57HDL	37.1 \pm 8.4	437 \pm 140
AIItrHDL	24.3 \pm 4.8	209 \pm 68

B_{max} , maximum binding. Values are means \pm SEM of five experiments performed with five different sets of HDL. Two-way ANOVA was used to compare apparent B_{max} of ligands in five experiments each comprising triplicate dishes: apoA-II effect, $P = 0.006$; experimental effect, $P = 0.004$. For B_{max} : AII-/-HDL versus AIItrHDL, $P = 0.016$; AIItrHDL versus C57HDL, $P = 0.040$; AII-/-HDL versus C57HDL, $P = 0.130$.

tween the association of the different ligands with CD36 were greater than the differences observed in the case of SR-BI (**Fig. 2**).

CD36-mediated selective uptake of CE from the three HDLs is shown in **Fig. 4B**. As for receptor-specific ligand association, marked differences in selective CE uptake were observed between the different HDLs, with the greatest selective uptake exhibited by AII-/-HDL and the least by AIItrHDL. At a ligand concentration of 60 $\mu\text{g/ml}$, selective uptake from AII-/-HDL exceeded that from AIItrHDL by ~3.5-fold and significant differences were observed between each of the ligands. Therefore, to a large extent, differences in selective uptake reflected the differences in cell association of the three ligands.

To assess whether the observed differences in CD36 binding of the different HDLs were the result of the varying concentrations of apoA-II in HDL particles, we compared the interaction of CD36 in transfected COS cells with defined 100 Å reconstituted particles (rHDL) containing apoA-II or lacking apoA-II (19) (**Fig. 5**). These reconstituted discs contained either two molecules of apoA-I

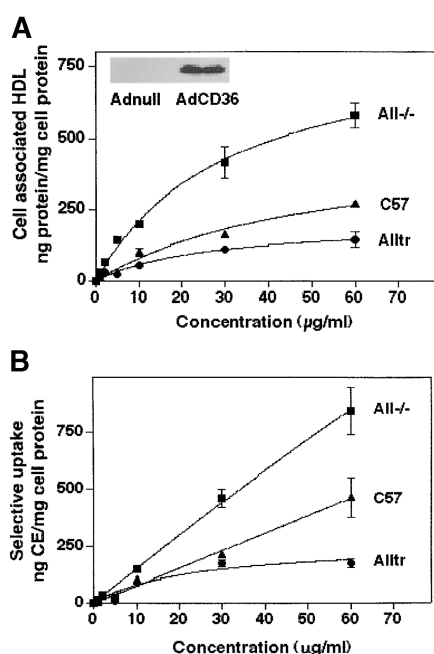


Fig. 4. Concentration-dependent association of mouse HDLs with mouse CD36. COS cells expressing mouse CD36 through adenovirus-mediated gene transfer were incubated for 2 h at 37°C with the indicated concentrations of $^{125}\text{I}/^3\text{H}$ -labeled HDLs, and the cell-associated label was quantified as described in Experimental Procedures. CD36-specific cell association values are shown and were calculated as the difference between values for cells treated with AdCD36 and Adnull. A: Cell-associated AII-/-HDL, C57HDL, and AIItrHDL. The inset shows Western blot analysis of the expression of CD36 in duplicate wells treated with Adnull or AdCD36 (10 μg protein/lane). B: Selective uptake of CE from AII-/-HDL, C57HDL, and AIItrHDL. Values are expressed as nanograms of CE and represent means \pm SEM of triplicate determinations. Two-way ANOVA was used to compare the selective uptake of CE at 60 $\mu\text{g/ml}$ in five experiments each comprising triplicate dishes: apoA-II effect, $P < 0.0001$; experimental effect, $P < 0.0001$. For selective uptake: AII-/-HDL versus C57HDL, $P < 0.0001$; C57HDL versus AIItrHDL, $P < 0.0001$; AII-/-HDL versus AIItrHDL, $P < 0.0001$.

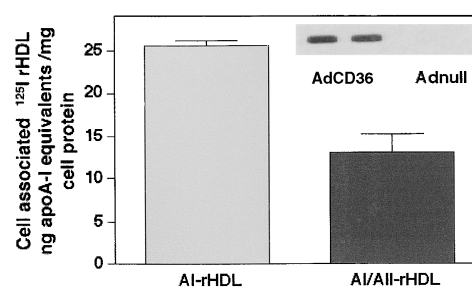


Fig. 5. Binding of rHDL to mouse CD36. COS cells expressing mouse CD36 through adenovirus-mediated gene transfer were incubated for 2 h at 37°C with 20 $\mu\text{g/ml}$ apoA-I equivalents rHDL. L- α -Palmitoylcholine-containing AI-rHDL and AI/AII-rHDL were iodinated in the protein moiety, and equivalent moles of each rHDL were added. Cell-associated label was quantitated as described in Experimental Procedures. Shown are CD36-specific cell association values calculated as the difference between values for cells treated with AdCD36 and Adnull. Values shown are means \pm SEM of quadruplicate determinations ($P = 0.0016$). The inset shows the expression of CD36 in duplicate wells treated with Adnull or AdCD36.

(AI-rHDL) or two molecules of apoA-I plus one molecule of dimeric apoA-II (AI/AII-rHDL). rHDL was added in the medium on the basis of equivalent particle numbers (expressed as apoA-I equivalents), and cell association was also expressed as apoA-I equivalents. As previously observed for SR-BI (19), the association of AI-rHDL to CD36 was significantly greater (~ 2 -fold) than that of AI/AII-rHDL. This result provided strong evidence that the apoA-II content of HDL influences HDL binding to CD36 and thus also selective uptake mediated by CD36.

Previous reports have shown that CD36 mediates selective uptake from HDL with a greatly reduced efficiency compared with SR-BI (23, 24, 42). The efficiencies with which CD36 mediated selective CE uptake from the different ligands were compared with SR-BI-mediated selective uptake in the same cell type (COS cells). In these experiments, COS cells expressed similar numbers (within 2-fold) of CD36 or SR-BI receptors on the cell surface, as judged by the binding of oxLDL, a common ligand. The efficiencies of receptor-mediated selective uptake were calculated as the amount of selective CE uptake relative to HDL associated with the cells. In Fig. 6, the efficiencies of CE uptake of the three ligands by CD36 and SR-BI were compared at two different ligand concentrations (5 and 60 $\mu\text{g}/\text{ml}$). In the case of SR-BI, no significant differences between the efficiencies of the three ligands were observed,

and efficiencies were similar at the two ligand concentrations. In the case of CD36, no significant differences were observed between the three ligands at either ligand concentration. However, selective uptake efficiency by CD36 for each of the HDL ligands was greater at the higher ligand concentration, such that the efficiency of uptake was ~ 2 - to 3-fold greater at the higher ligand concentration. This was consistent with the fact that selective uptake by CD36 occurred by a lower affinity process than HDL binding (Fig. 4). At a ligand concentration of 5 $\mu\text{g}/\text{ml}$, CD36 mediated selective uptake from each of the three HDLs with a significantly reduced efficiency compared with SR-BI (Fig. 6A). However, at the higher ligand concentration (60 $\mu\text{g}/\text{ml}$), the efficiency of uptake from each of the HDLs by CD36 was similar to that of SR-BI uptake (Fig. 6B).

DISCUSSION

In this study, we have investigated the influence of apoA-II on HDL binding and selective lipid uptake by two closely related class B scavenger receptors, SR-BI and CD36. The main approach was to assess the activity of three naturally occurring mouse ligands that differ in their content of mouse apoA-II as a result of either gene knockout or gene overexpression in transgenic mice. In the case of SR-BI, cell association and selective lipid uptake were inversely correlated with the apoA-II content of HDL particles. Differences between the ligands in SR-BI binding and selective uptake provide an explanation for the altered plasma HDL levels, HDL size, and clearance rates that are found in apoA-II null mice and apoA-II transgenic mice (9, 38). Marked effects of apoA-II on HDL binding by CD36 were observed, with apoA-II-deficient HDL exhibiting significantly greater binding and selective uptake than apoA-II-enriched HDL. Interestingly, this selective uptake process was dependent on a lower affinity process than HDL association, such that at higher ligand concentrations the efficiency of selective uptake from the different mouse HDLs reached levels similar to those observed for SR-BI-mediated selective uptake.

The effect of apoA-II on SR-BI binding of HDL and selective uptake is in agreement with earlier studies indicating that apoA-II exerted a negative effect on SR-BI-specific binding (17, 19) and on selective uptake in some (17, 18) although not all (19) reports. The molecular mechanism underlying this effect of apoA-II is not understood. We and others (39, 40) have previously shown greater binding and selective uptake of HDL₂ than HDL₃ particles by SR-BI, suggesting that particle size exerts a significant influence on SR-BI association of HDL. In the present study, however, particle binding was inversely related to particle size, with the greatest binding exhibited by AII $^{-/-}$ -HDL, the smallest of the HDLs tested. Another possibility is that the observed differences are attributable to a decreased apoA-I content in particles. However, evidence suggests rather that apoA-II in HDL results in a conformational change in apoA-I that alters its ability to bind SR-BI. Re-

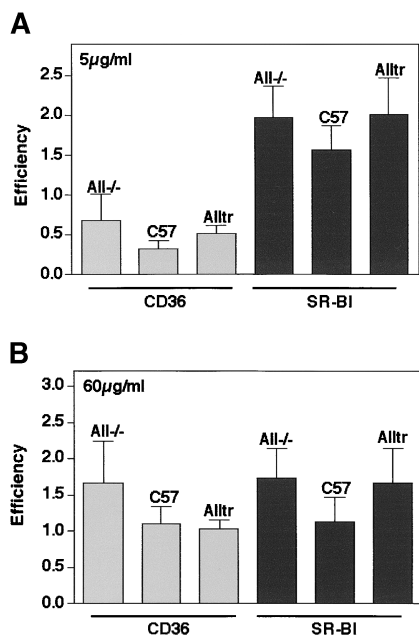


Fig. 6. Selective uptake efficiency of CD36 and SR-BI. The efficiency of selective uptake of HDL-CE at 5 $\mu\text{g}/\text{ml}$ (A) and 60 $\mu\text{g}/\text{ml}$ (B) by COS cells expressing mouse CD36 or mouse SR-BI through adenovirus-mediated gene transfer was calculated as nanograms of selective CE uptake per nanogram of cell-associated HDL protein. Values represent means \pm SEM of three experiments performed with three sets of ligands. Data were analyzed by paired *t*-test. CD36 efficiency at 5 $\mu\text{g}/\text{ml}$ versus 60 $\mu\text{g}/\text{ml}$ HDL: AII $^{-/-}$ -HDL, $P = 0.044$; C57HDL, $P = 0.049$; AIItrHDL, $P = 0.015$. Efficiency of CD36 versus SR-BI at 5 $\mu\text{g}/\text{ml}$: AII $^{-/-}$ -HDL, $P = 0.021$; C57HDL, $P = 0.015$; AIItrHDL, $P = 0.051$.

duced binding of rHDL containing varying amounts of apoA-II but similar amounts of apoA-I was shown by us for SR-BI (19) and in this study for CD36. The addition of apoA-II to rHDL, in fact, has been shown to alter the conformation and stability of lipid-bound apoA-I (43). It is interesting that differences in binding between the different mouse HDLs were largely a function of differences in the B_{max} of these ligands rather than differences in binding affinities. Similar observations were made in relation to SR-BI binding of rHDL particles (19). The explanation for this phenomenon is not understood. Recent evidence suggests the presence of more than one apolipoprotein binding site on SR-BI (15, 44, 45) and to the concept that a "productive complex" between receptor and ligand is necessary for selective uptake (46, 47). It is possible that different binding sites on SR-BI may be involved in the binding of HDLs that differ in their apolipoprotein composition, thereby accounting for the differences in B_{max} values observed for the different ligands. It should also be noted that HDL binding does not necessarily result in efficient selective uptake. Thus, chemical compounds that inhibit SR-BI-specific selective lipid uptake from HDL enhanced the binding of HDL to SR-BI (47). Furthermore, the in vitro enrichment of HDL with apoA-II resulted in increased binding but reduced selective lipid uptake by SR-BI in an adrenal cell line (18). It has also been shown that although SR-BI binds apoA-I-deficient HDL with high affinity, it does not mediate efficient selective lipid uptake (46).

Our finding of an increased rate of selective CE uptake from AII-/-HDL by SR-BI provides an explanation for the increased FCR of HDL and in part for the low levels of HDL observed in apoA-II-deficient mice. ApoA-II-deficient mice are characterized by HDL levels that are ~3-fold lower than those in control mice as a result of an increased FCR of both HDL CE (~1.7-fold) and protein (~1.8-fold) and a decreased rate of HDL production compared with that in control animals (9). An increased rate of selective CE uptake from AII-/-HDL by SR-BI expressed in the liver would contribute significantly to such differences. Increased selective lipid uptake by the liver would be expected to promote reverse cholesterol uptake, thereby providing a rationale for the antiatherogenic potential of HDL from apoA-II null mice. The explanation for the increased FCR of the protein component of AII-/-HDL is not known, and the mechanism responsible for apoA-I clearance in both normal and apoA-II-deficient mice remains unclear. The observed increase in selective CE from AII-/-HDL may also explain the decreased CE content found in these particles. Earlier studies also indicated that apoA-II in the mouse exerts an inhibitory effect on hepatic lipase and that increased lipase activity in apoA-II null mice contributes to a significant degree to reduced HDL levels in these mice (28).

In contrast to apoA-II-deficient mice, transgenic mice expressing mouse apoA-II exhibit increased plasma HDL levels, HDL size, and CE content (10, 13, 48). These features are in agreement with the decreased HDL selective lipid uptake we observed, although HDL turnover studies

have not been performed in these animals. The finding that increased apoA-II levels are associated with decreased selective lipid uptake is compatible with the model that apoA-II functions in a proatherogenic manner (8, 10–12) by inhibiting reverse CE transport to the liver. Other potential mechanisms by which apoA-II may retard reverse cholesterol transport include its effect on modulating the interaction of HDL with lipid transfer proteins and enzymes [reviewed in ref. (6)].

The binding of HDL was examined in COS cells overexpressing CD36 through adenovirus-mediated gene transfer (24). Interestingly, attempts to use stably transfected CHO cells expressing functional CD36 were unsuccessful. Despite overexpressing CD36 in significant amounts and in an apparently mature glycosylated 80 kDa form, transfected CHO cells failed to exhibit any significant receptor-dependent binding of either oxLDL or HDL, two known ligands for CD36. Two possible explanations are that receptors fail to reach the surface, despite undergoing apparently normal glycosylation, or that the conformation or localization of CD36 on the cell surface is atypical in these cells and prevents ligand recognition. In COS cells, expressed CD36 receptors exhibited high-affinity binding of each of the mouse ligands, with no significant differences between the affinities for the different ligands. However, significant differences in binding were observed in the B_{max} values of the ligands, with the level of binding being negatively correlated with the level of apoA-II in the particle. The explanation for the differences in B_{max} is not yet understood. As for SR-BI, this may be attributable to the presence of more than one type of apolipoprotein binding site on CD36.

CD36 was shown to mediate the uptake of CE from the three HDLs in a selective manner. Significant differences in selective uptake rates were found between the ligands, and these largely reflected differences in ligand association with CD36, with AII-/-HDL delivering greater amounts of CE compared with control or AIItrHDL. As previously reported (23, 24), at lower concentrations of ligand, CD36 mediated selective uptake from HDLs with an efficiency (selective uptake expressed as a function of HDL association) that was ~5-fold lower than that of SR-BI. However, CD36-mediated selective uptake appeared to depend on a lower affinity process than SR-BI binding, so that the efficiency of selective uptake at higher ligand concentrations actually approached that of SR-BI. The explanation for this divergence between the apparent affinities for the HDL association and selective uptake processes is not clear and requires a better understanding of the mechanisms involved in both the HDL cell association step and the subsequent selective CE transfer step that together determine the rate of selective uptake. The physiological significance of this low-affinity CD36-mediated uptake process is not yet clear.

The marked difference in SR-BI and CD36 binding of HDLs expressing varying amounts of apoA-II raises the interesting possibility that such ligands may also vary in their effects on processes affected by HDL interaction with cells. HDL triggers a wide variety of intracellular signaling

events in cells (49, 50), including the activation of various phospholipases, protein kinase C, mitogen-activated protein kinase (MAPK), heterotrimeric G-proteins, and tyrosine kinase. For instance, HDL binding to SR-BI activates Ras and subsequently the MAPK signaling cascade (51). HDL binding was also demonstrated to activate nitric oxide synthase in an SR-BI-dependent manner (52) that was shown to be mediated by estradiol associated with HDL (53).

CD36 is localized on plasma membranes within caveolae where it might serve as a targeting receptor for ligands, as a signaling molecule, or as a regulator of caveolar function. HDL is a high-affinity ligand for CD36, and its interaction with CD36 might induce cellular responses as well as possibly serve as a competitor for other CD36 ligands, thus modulating their effects. Selective uptake of lipids from HDL by CD36 may modify the cholesterol- and sphingolipid-rich caveolar structure and its functional role. Interestingly, strong similarities exist between the phenotypes of CD36 null mice and apoA-II transgenic mice, both of which have increased plasma HDL cholesterol, triglycerides, and free fatty acids and altered glucose metabolism. (25, 38). It has been hypothesized that apoA-II alters the composition of HDL such that HDL interaction with CD36 is impaired, leading to decreased fatty acid utilization in muscle and consequently increased insulin resistance in muscle (27). Our current findings, which demonstrate marked differences between apoA-II-rich and apoA-II-depleted mouse HDL, provide support for this idea.

In conclusion, our results demonstrate that HDLs containing different amounts of apoA-II exhibit marked differences in their interaction with the class B scavenger receptors SR-BI and CD36 such that the level of apoA-II in HDL is inversely correlated with HDL binding and selective CE uptake.

This work was supported by National Institutes of Health Grant HL-63763 (to D.R.v.d.W.). The authors thank Nathan Whitaker for excellent technical support.

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